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STUDIES ON SECONDARY TETANUS IMMUNIZATION IN MICE

II. Standardization of Tetanus Toxoid in Minimally Sensitized Mice[1]

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Mouse and guinea pig are the two species of animals generally employed for the bio-assay of tetanus toxoid. Unsensitized animals are used as a rule. Techniques of assaying tetanus toxoid against a standard preparation in unsensitized mice (mouse primary, hereafter referred to as MP) were described by some authors (1, 2), but these methods are unsatisfactory because the dose-response line is easily influenced by factors such as the purity of toxoid (3), the presence of adjuvant (4), the strain of mouse (5, 6), and the seasonal variation of immunizability (6). Guineas pigs (guinea pig primary, GPP) were shown to give more reliable results (4), but the prohibitive cost of maintaining a big colony renders it unfeasible as a routine procedure for most laboratories.

In previous experiments (7), observation was made that moderately sensitized mice, with circulating tetanus antitoxin levels around 0.004-0.1 iu/ml serum, were relatively insensitive to the variation of the size of booster dose. In contrast, both groups of minimally and strongly sensitized mice, with circulating antitoxin concentrations beyond the range mentioned above, responded sensitively to such variations. Accordingly, it was considered theoretically possible to assay tetanus toxoid in minimally sensitized mice (MSM). Interest of research along this line was further enhanced in view of the apparently unreasonable current practice of assaying tetanus toxoid in unsensitized animals by single injection. Prophylatics are generally evaluated by the criterion of antigenicity in primary immunization. Tetanus toxoids available nowadays could confer adequate protection in primary immunization only through multiple injections, therefore toxoid assay in its genuine sense should aim at testing the potency as a multiple-instead of single-injection antigen.

The experimental data presented herein will be dealt with in two parts. The first series of orientation experiments was done to work out the optimal conditions for obtaining an usable dose-response curve in MSM. The second series concerns the trial standardization of 3 lots of tetanus toxoids, which differed widely in physico-chemical properties, against a single standard with this newly proposed assay method. Additional potency tests were run also, with the same materials, with MP and GPP methods, and the results obtained are included for comparison.

MATERIALS AND METHODS

*Toxoids**. The plain toxoid LE202 was used in the orientation experiments, and the other toxoids in the standardization experiments. Toxoids were kept at 4-8 C during this study.

LE202 was a plain, monovalent product of ammonium sulphate purification which contained 0.963 mg total nitrogen, 0.79 mg trichloro-acetic acid precipitable nitrogen, and 1500 Lf per ml respectively. WHO-TEX-119 was a plain, monovalent product of alcohol purification which contained 1 iu/0.03 mg. It was first dissolved in a small amount of phosphate buffer, and then quickly mixed in 50% glycerin of phosphate buffer, pH 6.2, to the final concentration of 100 iu/ml for stock. 43J and TRC were $Al(OH)_3$ adsorbed preparations. The former was a diphtheria-tetanus combined vaccine purified by ultrafiltration, and was claimed by the producer to have a potency of 100 iu/ml by GPP method of

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* The toxoid LE202 was generously donated by Biologic Laboratories, Massachusetts Institute of Laboratories, Boston; 43J and TRC by Statens Seruminstitute, Copenhagen; 904-1 by Laboratory of Hygiene, Ottawa.

titration (8). The latter was a diphtheria-pertussis-tetanus combined vaccine purified by the double process of ultrafiltration plus ammonium sulphate salting-out, and was claimed to have a potency of 550 iu/ml by the same assay method. 904-1 was a plain, monovalent product purified by ultra-filtration. It was claimed by the original laboratory to have a potency of 52 iu/ml, but unfortunately no information was available to the author as to the method by which this toxoid had been assayed.

Challenging toxin and diluent. A single batch of dried tetanus toxin purified by ammonium sulphate and powder-diluted with glycine was used throughout. In orientation experiments with toxoid LE202, M/20 borate buffered saline containing 0.02% gelatin, pH 7.4, was used as the protective diluent for both toxoid and toxin. However, in later standardization experiments M/15 phosphate buffered saline was used instead, because it was found that suspensions of $Al(OH)_3$ adsorbed toxoids were relatively unstable in the borate buffer containing gelatin.

Animals. A free breeding strain of *balb* mouse, and an albino strain of guinea pig raised in the Laboratory were used. Female mice were used to avoid fighting, and guinea pigs were separated by sex to prevent mating. With the exception of Exp. No. 3, adequate planning was made so that the weight and cage distribution among experimental groups was comparable (3).

Injection, scoring and statistical treating. Injections were given subcutaneously with 0.5 ml volumes in mice, and with 1 ml volumes in guinea pigs, at the posterior upper part of the hind limb. The modified scoring scale of Wada *et al* (6) was adopted, because of its ability to give a slightly larger regression coefficient in MSM than the original scoring system of Ipsen (9, 13). Computation procedures recommended by Burn *et al* (10) were followed in calculating the regression coefficient and its error, and in testing the linearity of response lines. Parallelism between test toxoid and WHO standard was tested by t (11). ED_{50} was interpreted as the dose which produced mean score 3 obtained by computation.

RESULTS

Orientation Experiments

The optimal conditions of the size of sensitizing dose, the initial weight of mice, the time interval between secondary immuuization and toxin challenge (B-C interval), and the size of challenge dose for the MSM method of assaying tetanus toxoid were determined in the series of orientation experiments. It is conceivable that bizarre results were likely to occur in the earlier experiments, when the optimal conditions remained obscure one from the other.

The size of sensitizing dose (*Exp. 1, 2*). The mice used in Exp. 1 weighed 8–17 g with a mean of 11.9 g, while that in Exp. 2 ranged 10–17 g with a mean of 12.8 g. The majority of mice, *i.e.* about 3/4 in both experiments, fell within the weight range of 11–14 g. Groups of mice were sensitized with varying doses of tetanus toxoid, and then subgrouped to receive graded doses of secondary stimulus. For both experiments, the interval between primary and booster immunizations (P-B interval) was 28 days, and the B-C interval was 7 days. Five mld was used for challenge in each case.

Table I indicates that of the 3 sensitizing doses employed, the weakest one, *i.e.* 0.1 Lf, gave the best result, and that optimal sensitization can be induced by this dose. Sensitizing doses bigger than 0.1 Lf yielded, under the condition of the experiment, irregular response due to the strong residual immunity (Exp. 1-1), negative phase phenomenon (Exp. 2-1), or flattened slopes (Exp. 1-2 and 2-2). Of course the emergence of negative phase should have been avoided by prolonging the B-C interval, but it remains to be seen to what extent the irregular response and flattened slopes observed in the larger sensitizing dose groups could be improved by modifying the experimental procedure. The overall data are interpreted to mean that a state of minimal sensitization was generated by 0.1 Lf of tetanus toxoid, to account for the regular immune response to graded booster doses (7). This point was confirmed by a group of 12 mice which was bled for titration 20 days after the primary immunization of 0.1 Lf. Ten of these mice gave minimal circulating antitoxin titers not exceeding 0.002 iu/ml, while only 2 animals had supraminimal titers of 0.008 and 0.128 iu per ml respectively.

The error of the regression coefficient s^2 was large in all cases. The F values were fairly large too, *i.e.* all values exceeded 1. The data give no hint as to whether these were due to the use of relatively small mice, or to a combination of some other factors. The chance of departure from linearity was smaller in 0.1 Lf group than that in the groups sensitized with larger doses, since F ratios tended to become smaller as the sensitizing dose decreased.

The initial weight of mice (*Exp. 3*). In order to solve whether the initial weight of mice exerts

TABLE I

Determination of optimal experimental conditions for MSM method of assaying tetanus toxoid

Exp. no.	Mice			Sensitizing dose, Lf	P-B interval*, days	Booster		B-C interval**, days	Challenge dose, mld	Slope			ED_{50} Lf
	Weight range, g	Mean wt, g	No.			No. doses	Fold interval			b	s^2	F	
1-1	8-17	11.9	40	1.00	28	4	3.3	7	5	Irregular response			
1-2	8-17	11.8	38	0.33	28	4	3.3	7	5	1.238	5.16	2.35	0.005
1-3	8-17	11.9	40	0.10	28	4	3.3	7	5	1.999	6.08	1.04	0.023
2-1	10-17	12.8	40	1.00	28	4	3.3	7	5	Negative phase phenomenon			
2-2	10-17	12.8	40	0.33	28	4	3.3	7	5	0.424	6.91	3.69	0.039
2-3	10-17	12.8	39	0.10	28	4	3.3	7	5	2.520	5.31	2.78	0.034
3-1	11-13	12.2	40	0.33	38	4	3.3	7	5	1.306	5.63	2.91	0.010
3-2	14-16	15.1	40	0.33	38	4	3.3	7	5	2.199	7.07	0.22	0.070
3-3	17-19	18.0	35	0.33	38	4	3.3	7	5	1.049	4.19	0.76	0.010
4-1	12-15	12.7	39	0.10	30	4	3.3	4	5	Dose-response undetectable			
4-2	12-15	12.7	40	0.10	30	4	3.3	6	5	0.419	3.47	1.26	0.560
4-3	12-15	12.7	39	0.10	30	4	3.3	8	5	0.873	5.65	0.09	0.020
5-1	11-15	12.4	38	0.10	30	4	3.3	7	5	1.295	2.94	0.37	0.073
5-2	11-15	12.4	37	0·10	30	4	3.3	9	5	0.805	2.39	6.34	0.021
5-3	11-15	12.4	37	0.10	30	4	3.3	13	5	2.452	4.41	1.27	0.041
6-1	12-17	13.8	40	0.10	30	4	3.3	7	7	1.204	5.11	1.39	0.323
6-2	12-17	13.8	40	0.10	30	4	3.3	10	7	2.437	3.31	0.45	0.045
6-3	12-17	13.8	40	0.10	30	4	3.3	13	7	1.996	3.84	0.15	0.029
7-1	13-17	14.5	39	0.10	30	4	3.3	10	5	1.612	5.72	0.51	0.079
7-2	13-17	14.5	38	0.10	30	4	3.3	14	5	1.051	3.89	1.95	0.103
7-3	13-17	14.4	38	0.10	30	4	3.3	10	20	2.359	4.84	1.32	0.162
7-4	13-17	14.6	39	0.10	30	4	3.3	14	20	0.764	6.26	0.71	1.840
8-1	13-17	14.3	32	0.10	30	4	5.0	10	5	1.645	4.11	0.99	0.145
8-2	13-17	14.3	31	0.10	30	4	5.0	10	20	2.384	5.07	1.02	0.320

* P-B interval: interval between primary and booster immunization.

** B-C interval: interval between booster immunization and challenge.

any influence on the slope of secondary tetanus immune response, mice were grouped by weight and tested. The animals were immunized when the dose for optimal sensitization was not yet known, hence the use of the relatively large sensitizing dose of 0.33 Lf. The P-B interval was prolonged to 38 days in an effort to minimize the hazard of excessive sensitization which might have been produced. The animals were challenged with 5 mld 7 days after booster injection.

Table I shows that the steepest immune slope occurred in the group of mice weighing 14-16 g. This group also yielded the smallest F (negligible chance of departure from linearity), although in accompaniment of a large s^2 (great error of the regression coefficient). The balance of evidence, therefore, is in favor of the initial weight range of 14-16 g. As mentioned earlier (9) and described again fully in the comparative study that follows below, the inherently great error of the regression coefficient in MSM is unavoidable. One would not gain too much by trying to belittle the s^2 in MSM, because such efforts are theoretically futile (7).

The choice of mice weighing 14-16 g for primary sensitization agrees well with sensible deduction. Smaller mice are delicate to handle, and there is economically no reason to use older mice if moderately weighed ones suit the purpose. Since the single experiment gave results agreeable with laboratory conveniency, no further experiment on this subject was done. It is infered, therefore, animals weighing as close as possible to the range of 14-16 g should be used in MSM experiments.

***The B-C interval* (*Exp. 4-7*).** Animals in these experiments were minimally sensitized with 0.1 Lf of toxoid, and given secondary immunization 30 days later. The B-C interval was varied from 4 to 14 days in the major groups to detect its influence on the response line. A weak challenge dose of 5 mld was used in most experiments, but 2 additional groups were included in Exp. 7 to receive 20 mld for comparison.

During the testing period of 4-8 days after

booster immunization in Exp. 4, the animals appeared to retain an average level of residual immunity, conferred by the primary immunization, represented approximately by mean score 2. As expected, the secondary response was not detectable in the group of mice challenged 4 days after booster injection: even those receiving the largest dose of 0.3 Lf appeared to respond indifferently. Signs of differential response to graded booster stimuli started to appear when animals were challenged 6 days after booster (Exp. 4-2), and the situation improved further when the B-C interval was lengthened to 8 days (Exp. 4-3). The ED_{50} of the former group was also much larger than that of the latter. These findings, coupled with the irregular response and negative phase phenomenon observed in Exp. 1 and 2, indicate the hazard to challenge mice within 6 days after booster injection.

In Exp. 5, 6 and 7, the optimal B-C interval was tested at 7, 9-10 and 13-14 days. The mice in Exp. 5 and 6 challenged on the 13th day after booster gave slightly steeper slopes than those challenged on the 7th day. The ED_{50} of the former group was also comparatively smaller, and this was particularly eminent in Exp. 6. In this experiment, the ED_{50} of mice challenged on the 7th day after booster was 11.1-fold greater than those challenged on the 13th day, indicating that the secondary immune response was not fully developed in mice 7 days after booster injection.

In view of the slightly steeper slopes it yielded in Exp. 6 and 7, the 10-days-B-C-interval appears to give better result than 13-14 days. This is especially significant in Exp. 7-3 and 7-4 which yielded a 3.1-fold difference of b values between the groups, under the influence of a large challenge dose. Dropping of the group immunity constitutes another hazard to challenge the mice as late as 14 days after booster injection: in face of a large challenge dose, these mice produced an ED_{50} figure 11.4-fold larger than those challenged on the 10th day (Exp. 7-4 *versus* 7-3).

The size of challenge dose (*Exp. 7, 8*). Mice minimally sensitized with 0.1 Lf of toxoid in these experiments were boostered 30 days after the primary sensitization. Since earlier experiments revealed characteristically flattened slopes of secondary immune response in MSM, the booster dose interval in Exp. 8 was extended to 5-fold in an effort to cover maximum and zero responses. The results obtained in Exp. 7-2 and 7-4 will not be compared, because the long B-C interval of 14 days in these experiments has already been shown to be unfavorable.

Table I shows that modification of the booster dose interval did not affect the overall results, *i.e.* the figures of b, s^2, F and ED_{50} of 5 and 20 mld groups in Exp. 7-1 and 7-3 are comparable to the corresponding figures of the respective groups in Exp. 8. In both experiments, the 20 mld group yielded steeper slope than the 5 mld group. This is due to the ability of the larger challenge dose to bring out the near-zero response in the weakly boostered mice, provided that the dilution scale were well chosen. It will be noted that in both experiments, a 4-fold difference of the size of challenge dose resulted in only about a 2-fold difference of ED_{50}.

A large challenge dose possesses the additional theoretical merit to wipe out the individual variation of toxin susceptibility of the test animals (12). Since the experimental findings do not contradict theoretical considerations, a large challenge dose is considered preferable to small one.

Secondary tetanus response at different date after minimal sensitization (*Exp. 9, 10*). It is worthwhile to study whether significant change of secondary response patterns occurs as the time elapses after minimal sensitization. Negative evidence of change would favor the adaptation of MSM method of assaying tetanus toxoid as a practical laboratory routine: surplus animals can be 'prepared' ahead and made available for ready use at any later date when necessity arises.

Animals in these experiments were sensitized with 0.2 Lf of toxoid and were challenged with 5 (Exp. 9) and 10 (Exp. 10) mld respectively 10 days after secondary immunization. Table II shows that in Exp. 9, extension of the P-B interval did not influence adversely the general results. As a matter of fact, all figures of the steepness of the slope (b), the error of the regression coefficient (s^2), the linearity (F) and the group immunity (ED_{50}) appeared to improve as the P-B interval prolonged.

Exp. 10 yielded different results in that both figures of b and F suffered slightly as the P-B interval prolonged. Since similar variations were not encountered in the duplicate experiment, and the differences were by no means significant after all, one is led to speculate whether these were chance happenings conditioned by a state of supra-minimal sensitization in face of a relatively weak challenge. Definite conclusion is not available pending further experimentation, but the overall data in Table II indicate that for practical purposes, immunologically acceptable dose-response curves could be obtained within the P-B interval of 15-71 days.

TABIE II

Secondary tetanus response at different date after min'mal sensitization

Exp. no.	Mice: Weight, g Range	Mice: Weight, g Mean	No.	P-B interval, days	Booster dose, Lf	Result after challenge: Days dead 1	2	3	4	5	6	7	Signs of survivals at 7th day +	—	Mean score	Slope b	Slope s^2	Slope F	ED_{50} Lf
9-1	13-16	14.0	10	17	1.0000		1				1		3	5	4.50	0.980	6.74	0.61	0.090
			10		0.2500		3	2	1				1	3	2.70				
			10		0.0625		3	2					2	3	2.80				
			10		0.0156		5	1						4	2.50				
9-2	13-16	14.0	10	37	2.0000			2					1	7	4.80	1.454	5.20	0.49	0.037
			10		0.4000			2					2	6	4.60				
			10		0.0800		1	3	1				1	4	3.40				
			9		0.0160		3	4						2	1.78				
9-3	13-16	14.0	10	71	0.5000			1				1	1	7	5.00	1.488	3.83	0.21	0.030
			10		0.1000			3	3					4	3.60				
			10		0.0200		1	4	2		1			2	2.50				
			10		0.0040			7	1			1		1	1.90				
10-1	13-17	14.1	10	15	0.3300		1						1	8	5.20	2.618	6.04	0.06	0.050
			10		0.1000		3						2	5	3.80				
			10		0.0330		5	1					1	3	2.30				
			10		0.0100		7			1			1	1	1.30				
10-2	13-17	14.1	9	30	2.0000			1						8	5.44	1.117	5.28	0.78	0.008
			10		0.4000			2				1		7	4.70				
			10		0.0800			2						8	4.80				
			9		0.0160		2	3					1	3	2.78				
10-3	13-17	14.1	10	62	0.5000							2	1	7	5.20	1.731	5.19	1.85	0.027
			10		0.1000		3	1	1					5	3.40				
			10		0.0200		2	1					2	5	3.90				
			10		0.0040		7	1	1					1	1.00				

In summary, the orientation experiments revealed the following optimal conditions for obtaining an usable secondary tetanus response curve in mice. ***Minimal sensitization*** should be induced in mice weighing ***13-17*** g, and the animals should preferably be tested with a ***large challenge dose*** of about 20 mld, at about ***10 days after secondary immunization.*** Given optimal experimental conditions, the mice are expected to be usable for titration any time about 15-71 days after minimal sensitization, and the chance of departure from linearity of the response line is usually small. However, even with the combination of all optimal experimental conditions, a small regression coefficient with rather large error is likely to be obtained. The flattening of the response curve could be compensated somewhat by adopting a widely spaced booster dose scale, *e.g.* 5 to 10-fold dose interval, to cover maximum and zero responses. Increase of the number of test animals, on the other hand, appears to be a remedy to minimize the inherently great error of the regression coefficient.

Standardization Experiments

A total of 10 assay experiments were conducted. 6 MSM (Table III), 2 MP and 2 GPP (Table IV) experiments were performed on test toxoids 43J and TRC. Due to the insufficient amount of toxoid 904-1 available to the author, GPP test was not conducted for this toxoid and only 3 MSM experiments were run. With the exceptions of Exp. 13 and 14, which were done in November-December 1961, the other experiments were carried out under the warm weather condition of March-July at the same year. The results obtained by different assay methods are described below.

Regression coefficient. As shown in Table V, the steepness of the response curve as represented by the regression coefficient b was in the decreasing order of GPP>MP>MSM. This was true in all the three toxoids tested, regardless of physico-chemical properties and potency. The regression coefficient multiples between different animals can be conveniently expressed as follows:

TABLE III

Standardization of tetanus toxoid in minimally sensitized mice

Experiment		Mice	Toxoids	Booster	Result after challenge								Mean	Slope			ED_{50}*
					Days dead						Signs of survivals at 7th day						
Conditions	No.	no.		dose*	1	2	3	4	5	6	7	+	−	score	b	s^2	F	
Sensitizing dose 0.3 Lf, P-B interval 48 days, challenge dose 20 mld.	11	11	WHO-TEX 119	3.00000									11	6.00	1.298	5.62	0.52	0.02460
		11		0.75000		1	1						9	5.00				
		11		0.18800		3	1					3	4	3.36				
		11		0.04690		4		1					6	3.55				
		11		0.01172		4	1			1	1		4	2.82				
		11	43J	0.016667									11	6.00	1.858	4.85	1.11	0.000267
		11		0.004167							1		10	5.73				
		11		0.001042		2	1			1		1	6	4.00				
		11		0.000261		7						1	3	2.00				
		11		0.000065		6	1						4	2.27				
		11	TRC	0.002500		2							9	4.91	1.135	7.80	0.52	0.000056
		11		0.000630		1	1	1					8	4.73				
		11		0.000156		5	1						5	2.82				
		11		0.000039		5	1						5	2.82				
		11		0.000010		7							4	2.18				
		11	904-1	0.025000		3						1	7	4.18	1.298	7.13	1.68	0.001380
		11		0.006250		1		1				1	8	5.00				
		11		0.001560		5	1						5	2.82				
		11		0.000390		8	1						2	1.18				
		11		0.000100		7							4	2.18				
Sensitizing dose 0.2 Lf, P-B interval 31 days, challenge dose 15 mld.	12	10	WHO-TEX 119	3.0000								3	7	5.40	1.502	5.36	0.76	0.0701
		10		0.6000		2	1					1	6	4.10				
		10		0.1200		1	2		1				6	4.10				
		10		0.0240		6		1				1	2	1.90				
		10	43J	0.016667								2	8	5.60	2.518	4.64	0.43	0.001425
		10		0.003333		2	1						7	4.30				
		10		0.000667		6	1					1	2	1.70				
		10		0.000133		9							1	0.60				
		10	TRC	0.005000			1					2	7	5.10	1.396	6.60	0.99	0.000112
		11		0.001000		3						4	7	4.18				
		10		0.000200		2	1						7	4.30				
		10		0.000040		7							3	1.80				
Sensitizing dose 0.1 Lf, P-B interval 20 days, challenge dose 15 mld.	13	10	WHO-TEX 119	3.0000		1	1			1		1	6	4.40	1.884	4.37	0.19	0.4764
		11		0.3000		3	2	1				2	3	2.82				
		11		0.0300		8	1	1		1				0.64				
		10	43J	0.016667		1	1					1	7	4.67	1.949	4.01	7.91†	0.005430
		11		0.001667		9	1						1	0.64				
		10		0.000167		7	2						1	0.80				
		10	TRC	0.016667									10	6.00	2.899	2.51	0.54	0.001358
		11		0.001667		2	2				1	1	5	3.55				
		10		0.000167		8	2							0.20				

TABLE III *Continued*

Experiment		Mice	Toxoids	Booster	Result after challenge								Mean	Slope			ED_{50}*
					Days dead							Signs of survivals at 7th day					
Conditions	No.	no.		dose*	1	2	3	4	5	6	7	+	−	score	b	s^2	F	
Sensitizing dose 0.2 Lf, P-B interval 31 days, challenge dose 15 mld.	14	12	WHO	3.0000			1					2	9	5.25	1.138	6.18	0.73	0.0789
		14	TEX	0.3000		4	2					4	4	3.00				
		15	119	0.0300		7	1	1					6	2.67				
		14		0.0030		10					1		3	1.50				
		14	43J	0.016667									14	6.00	1.621	4.61	0.78	0.000574
		13		0.001667		5	2	1					5	2.69				
		15		0.000167		7	3						5	2.20				
		14		0.000017		8	5						1	0.79				
		15	TRC	0.016667									15	6.00	1.627	3.96	0.92	0.000213
		14		0.001667		2						2	10	4.86				
		14		0.000167		4	5	1					4	2.29				
		14		0.000017		7	4					1	2	1.43				
Sensitizing dose 0.1 Lf, P-B interval 18 days, challenge dose 15 mld.	15	9	WHO-TEX	3.0000								2	7	5.56	1.733	4.54	0.55	0.0592
		9		0.6000								3	6	5.33				
		9	119	0.1200		3						2	4	3.56				
		9		0.0240		5	1						3	2.11				
		8	43J	0.016667									8	6.00	1.695	3.35	1.67	0.000203
		9		0.003333								1	8	5.78				
		9		0.000667		2	1				1	3	2	3.11				
		8		0.000133		2	2					1	3	3.00				
		9	TRC	0.005000					1				8	5.67	2.272	3.76	1.97	0.000177
		9		0.001000					1		1		7	5.33				
		9		0.000200		3						1	5	3.78				
		9		0.000040		6	2						1	0.89				
		9	904-1	0.016667		3	1					1	4	3.22	1.227	6.99	2.31	0.003390
		9		0.003333		2						1	6	4.44				
		9		0.000667		7						1	1	1.11				
		8		0.000133		6							2	1.50				
Sensitizing dose 0.1 Lf, P-B interval 23 days, challenge dose 20 mld.	16	10	WHO-TEX	1.000		2	1					4	3	3.50	1.674	5.60	2.64	0.161
		10		0.200		2						1	7	4.60				
		10	119	0.040		7						1	2	1.60				
		10		0.008		9							1	0.60				
		9	43J	0.006250		1						1	7	5.11	2.004	4.74	2.48	0.001275
		10		0.001250		4	1					3	2	2.50				
		10		0.000250		9							1	0.60				
		10		0.000050		8						1	1	1.00				
		9	TRC	0.005000									9	6.00	2.213	5.63	1.61	0.000494
		10		0.001000		4	1					1	4	2.90				
		10		0.000200		7						1	2	1.60				
		10		0.000040		8							2	1.20				
		9	904-1	0.016667		3	1					1	4	3.22	1.312	4.43	1.07	0.028050
		10		0.003333		8						1	1	1.00				
		9		0.000667		7						1	1	1.11				
		10		0.000133		10								0				

* Expressed in terms of iu for WHO-TEX 119, ml for the other toxoids.

† Significant departure from linearity at 0.01% probability level.

TABLE IV

Protocol of tetanus toxoid standardization in mouse and guinea pig by primary immunization

Exp. no.	Animal			Toxoid		Challenge		Result after challenge									Slope			ED_{50}*
	Kind	Weight range, g	No.	Lot	Dose*	Days after immunization	dose, mld	Days dead							Signs of survivals at 7th day		Mean score	b	s^2	F	
								1	2	3	4	5	6	7	+	−					
17	Mouse	17–22	10	WHO-TEX 119	10.000	15	20		1						1	8	5.20	2.691	3.46	2.63	0.815
			10		2.500							2			1	7	5.20				
			10		0.625				3	1					3	3	3.10				
			10		0.156				8	1					1		0.50				
			10	43J	0.05000											10	6.00	4.984	2.44	0	0.00313
			10		0.01250											10	6.00				
			10		0.00313				4						3	3	3.00				
			10		0.00078				10								0				
			10	TRC	0.01667											10	6.00	2.988	3.20	2.54	0.00031
			10		0.00417											10	6.00				
			10		0.00104					1					1	8	5.30				
			10		0.00026				5						3	2	2.40				
			10	904-1	0.05000					1					4	5	4.70	2.492	1.52	10.2†	0.02500
			10		0.01250				7		1				2		1.10				
			10		0.00313				8	2							0.20				
			10		0.00078				10								0				
18	Mouse	18–23	10	WHO-TEX 119	4.000	16	20								2	8	5.60	3.158	4.55	0.93	0.729
			10		1.000				2	3					2	3	2.90				
			10		0.250				5	2					1	2	1.80				
			10	43J	0.01000											10	6.00	3.987	3.11	3.58	0.00138
			10		0.00333				1							9	5.40				
			10		0.00111				5						4	1	2.20				
			10	TRC	0.00125				1						2	7	5.00	4.156	3.93	0.04	0.00043
			10		0.00031				5	1					1	3	2.30				
			10		0.00008				10								0				

TABLE IV *Continued*

Exp. no.	Animal: Kind	Animal: Weight range, g	Animal: No.	Toxoid: Lot	Toxoid: Dose*	Challenge: Days after immunization	Challenge: dose, mld	Result after challenge: Days dead 1	2	3	4	5	6	7	Signs of survivals at 7th day: +	—	Mean score	Slope: b	s^2	F	ED_{50}*
			10	904-1	0.05000					1	2				4	3	4.10	3.407	1.38	13.12†	0.03450
			10		0.01250				9						1		0.40				
			10		0.00313				10								0				
			8	WHO-TEX 119	9.000											8	6.00	5.099	0.97	31.04†	0.557
			8		3.000											8	6.00				
			8		1.000										1	7	5.75				
			8		0.333				4	2			1		1		1.13				
			8	43J	0.10000											8	6.00	4.267	2.53	0.26	0.01760
19	Guinea pig	230–300	8		0.03330	20	20		1						3	4	4.50				
			8		0.01110				4		1				2	1	2.13				
			8		0.00370				8								0				
			8	TRC	0.01667										4	4	5.00	5.238	1.76	1.27	0.00805
			8		0.00556				2	3	2					1	1.88				
			8		0.00185				8								0				
			8		0.00062				8								0				
			8	WHO-TEX 119	3.000											8	6.00	5.107	1.09	18.00†	0.579
			8		1.000										2	6	5.50				
			8		0.333				5		1	1		1			1.13				
			8	43J	0.03333										4	4	5.00	4.707	2.19	0.24	0.01310
20	Guinea pig	230–300	8		0.01111	29	20		2	1	1				4	4	2.50				
			8		0.00370				7						1		0.50				
			8	TRC	0.01667							1		1	1	5	5.00	4.721	3.05	2.69	0.00769
			8		0.00556				4	2					1	1	1.50				
			8		0.00185				7						1		0.50				

* Expressed in terms of iu for WHO-TEX 119, ml for the other toxoids.

† Significant departure from linearity at 0.01% probability level.

TABLE V

Mean statistical figures obtained by different assay methods

	b				s^2				Potency iu/ml		
	43J	TRC	WHO	904-1	43J	TRC	WHO	904-1	43J	TRC	904-1
MSM	1.961	1.924	1.538	1.279	4.37	5.04	5.28	6.18	141	409	14
MP	4.481	3.572	2.925	2.950	2.78	3.57	4.01	1.45	503	2,268	28
GPP	4.487	4.980	5.103	—	2.36	2.41	1.03	—	37	75	—

	43J	TRC	WHO	904-1
MP/MSM	2.3	1.9	2.0	2.3
GPP/MSM	2.3	2.6	3.5	—
GPP/MP	1.0	1.4	1.8	—

It will be noted that the *b* ratios between MP and MSM lay in the fairly constant range of 1.9-2.3. However, in the group comparisons of GPP/MSM and GPP/MP, the ratios in 43J and TRC were smaller than those in WHO. This is interpreted to mean that the presence of aluminium adjuvant tended to minimize the difference of steepness of response curves obtained in different animals.

In both MSM and MP, the adsorbed toxoids 43J and TRC gave steeper response curves than the plain toxoids WHO and 904-1 (Table V). Our observations confirm earlier reports in that the slope difference between preparations is comparatively small in GPP (4). The average *b* values for plain toxoids WHO and 904-1, and adsorbed toxoids obtained by us in *balb* strain of mouse are 2.93 and 2.85, and 4.03 respectively. These come fairly close to the corresponding figures of 2.72 and 3.73 in *gpc* mouse reported by Murata *et al* (3).

Error of the regression coefficient. The error of the regression coefficient *b* is determined mainly by the average error variance for the total doses s^2, which measures also the extent of variation of the individual animal. Table V indicates that regardless of the kind of toxoids tested, GPP invariably gave the smallest s^2, MP the second, and MSM the largest. Strengthened by the additional ability to produce a steep response slope mentioned above, the GPP method emerges itself as best suited statistically for assaying tetanus toxoid, *i.e.* it is supposedly capable of detecting the slightest difference of potency with the greatest reliability. In contrast, the MSM method appears to be the least satisfactory statistically.

Ipsen found in MP that a reverse relationship existed between the steepness of the response slope and the extent of intra-assay error (13). In our case, out of 15+10=25 comparable animal-toxoid combinations shown in Table V, the majority of 22 gives the regular result that a steep regression line accompanies a small error of the slope. This relationship always holds true when different assay methods are compared by a single preparation. However, 3 exceptional cases occur when different preparations are compared by a single assay method (between 43J and TRC in GPP, 43J and 904-1 in MP, and TRC and 904-1 in MP).

The mean s^2 figures for plain toxoids WHO and 904-1 in *balb* mouse obtained in this study were 4.01 and 1.45 respectively. The former compares favorably with 3.71 in *gpc* mouse reported by Wada (6), but the latter falls short.

Linearity and parallelism. The linearity is tested by the variance ratio *F* between the mean squares of components 'deviations from regression' and 'within doses'. 11/21 of the *F* values shown in Table III are smaller than 1, indicating undoubtful linear relationship in these cases of MSM method of titration. In the remaining 10 cases where the first mean square exceeds the second, the *F* values of 9 are under 0.05% of probability and are therefore considered statistically insignificant. It is only in the single case of Exp. 13-2 that significant departure from linearity occurs.

On the other hand, 2/9 of GPP titrations and 2/8 of MP titrations depart significantly from linearity at 0.01% probability level, while the linearity of the rest remains statistically acceptable. It is interesting that all 4 cases of greater *F* belong to the plain toxoids WHO and 904-1. The greater frequency of large *F* values in both GPP and MP methods does not necessarily mean perhaps, as usually should, real departure from linearity, because of the following considerations. Firstly, the mean square within doses s^2 is smaller in both MP and GPP than in MSM. Accordingly, given the same mean square of deviations from regression, the variance ratio *F* is unavoidably larger in the former. Secondly, the steep response lines of both GPP and MP

renders it difficult to select an adequately narrow dilution interval to titrate around ED_{50}, the change of immunity score around which being most sensitive. The leveling-off effect near both extremes of maximum and zero responses is likely to occur under such conditions, giving rise to the impression of non-rectilinearity.

In all the three methods, the slopes of the response lines between test toxoid and standard can be considered parallel statistically in almost every case (Table VI). Toxoid 43J in Exp. 17 is the only exception. It is justifiable, therefore, to compute the potency of the test toxoid by a common slope shared with the standard.

Potency. The individual potency estimation records are given in Table VI, and the mean figures in Table V. It is remarkable that the superior antigenicity in the order of TRC>43J>904-1 was evidenced in every titration, despite the slight variation of potency ratios between the 3 toxoids in different experiments. The relative potency between the 3 toxoids appears to be closely comparable for different assay methods. Thus the mean potency ratios of TRC: 43J: 904-1 for GPP, MSM, and MP are 2.0:1.0:x, 2.9: 1.0:0.1, and 4.5:1.0:0.06 respectively. It is conceivably difficult to conclude from the limited number of experiments, but the available data in Table VI suggest that in GPP the fluctuation of relative potency was the least, accordingly reproducibility the greatest. In MSM, the potency multiple of TRC/43J varied between the narrw range of 1.7–3.8 in 5 out of 6 experiments, while Exp. 12 yielded a difference between the toxoids as big as 8.1-fold. The 2 ratio figures in MP eperiments differed 4-fold. The potency ratio 43J:904-1 varied to similar extents in different assay methods.

Although the relative potency between toxoids are closely comparable for different assay methods, the absolute values obtained by different methods

TABLE VI

Results of potency testing of tetanus toxoid by different methods

Exp. no.	Method	Toxoid	Parallelism* P	Common slope**	Potency†	
					iu/ml	TRC:43J:904-1
11	MSM	TRC	>0.5	1.216	360	2.2:1:0.11
11	MSM	43J	0.4–0.2	1.527	164	
11	MSM	904-1	>0.5	1.298	18	
12	MSM	TRC	>0.5	1.449	568	8.1:1
12	MSM	43J	0.2–0.1	2.010	70	
13	MSM	TRC	0.1–0.05	2.379	328	3.8:1
13	MSM	43J	>0.5	1.916	86	
14	MSM	TRC	0.4–0.2	1.394	440	3.2:1
14	MSM	43J	0.4–0.2	1.387	136	
15	MSM	TRC	0.5–0.4	2.002	464	1.7:1:0.06
15	MSM	43J	>0.5	1.714	280	
15	MSM	904-1	>0.5	1.480	16	
16	MSM	TRC	>0.5	1.943	296	2.7:1:0.08
16	MSM	43J	>0.5	1.839	110	
16	MSM	904-1	>0.5	1.493	9	
17	MP	TRC	>0.5	2.846	2,836	9.6:1:0.12
17	MP	43J	0.05–0.025	2.837	296	
17	MP	904-1	>0.5	2.592	34	
18	MP	TRC	0.4–0.2	3.657	1,700	2.4:1:0.03
18	MP	43J	>0.5	3.572	710	
18	MP	904-1	>0.5	3.282	21	
19	GPP	TRC	>0.5	5.134	74	2.5:1
19	GPP	43J	>0.5	4.683	30	
20	GPP	TRC	>0.5	4.915	75	1.7:1
20	GPP	43J	>0.5	4.908	44	

* t test. $t=\frac{b_1-b_2}{s_{b_1-b_2}}$

** Shares with WHO-TEX-119 justified by parallelism.

† Computed on the basis of common slope.

differ widely. Taking the potency data reported by the original laboratories as standard values, the ratios of different mean potency figures to the former are as follows:

	GPP	Reported	MSM	MP
43J	0.37 (37)	1 (100)	1.41 (141)	5.03 (503)
TRC	0.14 (75)	1 (550)	0.73 (409)	4.12 (2,268)
409-1	—	1 (52)	0.27 (14)	0.54 (28)

It will be seen that so far as adsorbed toxoids 43J and TRC are concerned, GPP produced the lowest, and MP the highest, potency figures when titrations were run against plain WHO standard. The mean potency obtained from these 2 methods differ as much as 13.8-fold in 43J, and 30.3-fold in TRC! The data are also self-evident in disclosing that the titration result of MSM comes closer to the reported figures than that of GPP, the very method, with slight modifications, through which the original potency figures were obtained in Copenhagen.

In the plain toxoid 904-1, both MSM and MP methods produced mean potency figures smaller than the value reported by the original laboratory. However, the MP figure is twice larger than MSM and approaches the reported value.

Immunizability. The computation of the approximate immunizability ratio involves calculation of the geometric mean ED_{50} first, and subsequent comparison of different animal-toxoid combinations on equal body weight basis. Thus the geometric mean ED_{50} of WHO standard toxoid in unsensitized *balb* mice with an average weight of 20 g was 0.771 iu. Assuming that the immunizability of MSM weighing 17 g was identical to that of MP with the same weight, then the expected ED_{50} for the former should be $(0.771 \times 17) \div 20 = 0.655$ iu, in contrast to the experimentally obtained figure of 0.0637 iu. Accordingly weight for weight, minimally sensitized *balb* are considered to be $0.655 \div 0.0637 = 10.3$-fold more responsive to WHO standard than unsensitized ones. The approximate immunizability ratios of different animal-toxoid combinations thus obtained are given in Table VII.

The comparison of GPP *versus* MP shown in Table VII reveals the interesting species difference of immunological response to plain tetanus toxoid. With the particular strains of animals we used, the mouse was about 16.5-fold less responsive to plain WHO toxoid than the guinea pig. On the other hand, differential species response to adsorbed toxoids was apparently nonexistent. These apparently controversial findings are interpreted to mean that antitoxin formation was promoted unequally by aluminum adjuvant in the two species. In other words, the aluminum gel produced much greater adjuvant effect (about

TABLE VII

*Approximate immunizability ratios of different animals computed on the basis of geometric mean ED_{50} and average body weight**

	Plain toxoids				Adsorbed toxoids			
	WHO		904-1		43J		TRC	
	Geom. mean ED_{50}, iu	Immun. ratio	Geom. mean ED_{50}, ml	Immun. ratio	Geom. mean ED_{50}, ml	Immun. ratio	Geom. mean ED_{50}, ml	Immun. ratio
GPP	0.5680				0.015200		0.007870	
MP observed	0.7710	−16.5		—**	0.002073	−1.6	0.000365	+1.8
expected	0.0468				0.001270		0.000656	
MP	0.7710		0.02900		0.002073		0.003649	
MSM observed	0.0637	+10.3	0.00508	+4.9	0.000560	+3.2	0.000152	+2.0
expected	0.6550		0.02465		0.001762		0.000310	
GPP	0.5680				0.015200		0.007870	
MSM observed	0.0637	−1.6		—**	0.000560	+1.9	0.000152	+3.7
expected	0.0402				0.001080		0.000557	

* Approximate average body weight used for computation:
Mouse primary (MP) 20 g
Mouse minimally sensitized (MSM) 17 g
Guinea pig primary (GPP) 240 g

** GPP titration was not conducted due to insufficient amount of toxoid.

16 times stronger in terms of ED_{50}) in poor responding species than in good one, so that the former was rendered possible to catch up the latter in its antitoxin producing ability.

The expression of the influence of minimal sensitization on the immunizability of mice differed, depending on the kind of toxoids used for testing. Thus MSM were found to be 4.9 to 10.3-fold more responsive than MP when plain toxoids were used for titration, but were only 2.0 to 3.2-fold more responsive when adsorbed toxoids were used. By virtue of the minimal sensitization, the wide gap between GPP and MP toward plain toxoid was brought down to the negligible difference of 1.6-fold between GPP and MSM. As a result, GPP and MSM possessed almost, though not quite, comparable immunizability to toxoids with widely different physico-chemical properties.

DISCUSSION

Greenberg reported a thorough study of assaying tetanus toxoids against a fluid standard (4). Where direct comparison of assay results on adsorbed toxoids were available, the MP method generally produced potency figures much higher than GPP. Inexplicable high results were occasionally obtained with the MP method, even when the most exact statistical criteria on slope comparison had been met satisfactorily. Similar results were obtained in this study, but it appears that at least partial answer lies in the incomparable immunizability of the two species of animals. In comparison with the guinea pig, the *balb* mouse was shown to be about 16-fold less responsive to fluid WHO standard toxoid, but as responsive to adsorbed toxoids. Accordingly, assaying an adsorbed toxoid against a fluid standard in MP would simulate the situation of measuring weights in a balance with wrong weight standards. Thus the absolute potency of an adsorbed toxoid (the weight of an object being measured) would be over-evaluated in MP (the balance), because more toxoid molecules in fluid standard were needed in MP, weight for weight, than in GPP to match the equivalent antigenicity of the adsorbed toxoid (degrading, consequently over-labeling of the surface value of weight standards to hit balance). The question of slope difference was definitely not involved in this regard, because in both Exp. 17 and 18, raising the slope values of fluid WHO standard to identical levels of adsorbed toxoids would increase further the potency figures instead of decreasing them.

The practice of assaying antigens against a single standard preparation demands the use of a stable testing system. The MP method of assaying tetanus toxoid has theoretical hazards in this regard, in view of the variation of antigenic compositions between different preparations, and the established fact that the immunologic reactivity of unsensitized mice is easily changed by various factors, such as the purity of toxoid (3), the presence of adjuvant (4, *vide supra*), the strain of mouse (5, 6) and the surrounding temperature (6, 14). Thus the test-toxoid may be crude or highly purified, monovalent or polyvalent, hence the possibility of antigenic competition or synergism (15) may be eithor exaggerated or overlooked in unsensitized mice. Valid assay can perhaps be obtained in unsensitized mice when adsorbed toxoids are titrated against an adsorbed standard, as shown by Ipsen (13), because the minor difference of antigenic make-up between preparations is likely dwarfed by the aluminum adjuvant (see GPP *vs* MP, MP *vs* MSM and GPP *vs* MSM in 43J and TRC, Table VII). In fluid *versus* fluid assays however, it pays to be cautious to use unsensitized mice as the test-animals because antigenic interactions may be exaggerated or overlooked, as the case may be.

Despite all these hazards, the mouse has distinct economical advantages over the guinea pig as a test-animal. Improvement of the mouse assay might be achieved by refining the MP technique (3, 5, 6, 14, 16), or by trying alternative approach such as the MSM method. The actual value of MSM method remains to be ascertained by further experimentation, but the available data presented here seem encouraging. In the majority of our assays with this method, the linearity and parallelism of dosage-response lines, the reproducibility of potency in both absolute and relative terms, and the correlation of potency with the other laboratories appear satisfactory. Apparently, the MSM method simulates the condition of actual use of toxoid in humans better than the MP method. The flattened slopes of dosage-response lines obtained by this method did not seriously affect the sensitivity of the assay, as evidenced by the reproducibility of potency figures. Instead, the flattened slopes can be used to advantage to catch the unknown ED_{50} of test-toxoids by covering a wide dilution range with relatively few animals. It may be argued that by the conventional statistical criterion the MSM method looks awkward, because the great error of the regression coefficient would cast serious doubt on the confidence limit of the

potency. It is our belief, however, that the concern of immunological, instead of statistical, soundness should come first in evaluating assay methods for antigenic materials. In this connection the observation of Greenberg (4) is relevent: statistically unacceptable assays might give correct results nevertheless, while occasional assays which met satisfactorily the most exact statistical criteria yielded results completely out of line.

The different immunizability within a given population can be smoothed out to some extent by a secondary antigenic stimulus (17), and here we witnessed another form of smoothing-out effect, *i.e.* the approximation of immunizability difference between two species, mouse and guinea pig, by minimal sensitization of the poorer responder. The fact that strikingly similar titration results were obtained with entirely different assay methods (GPP and MSM), performed in different laboratories, could hardly have been fortuitous, and it can be adequately explained by the smoothing-out effect. On the other hand, no proper interpretation is available to account for the discrepancy of titration results obtained with the same GPP method performed in two laboratories.

SUMMARY

Under the conditions of this experiment, the optimal conditions for assaying tetanus toxoid in minimally sensitized mice were determined and defined as follows: Mice weighing 13-17 g should be sensitized with about 0.1 Lf of toxoid to produce a minimal immunity represented by circulating antitoxin concentrations less than 0.004 u/ml of serum, and later challenged with large dose of toxin 10 days after the secondary immunization. The slope of dosage-response line thus obtained was flattened, and the error of the regression coefficient was rather large. However, the linearity and parallelism of dasage-response lines were generally acceptable, and the reproducibility of potency in both absolute and relative terms was satisfactory.

Three lots of foreign tetanus toxoids with widely different physico-chemical properties were assayed in minimally sensitized mice, unsensitized mice, and unsensitized guinea pigs for comparison. The potency figures obtained by the minimally sensitized mice method agreed with the reported values better than those obtained by the other methods.

The problem of immunizability in relation to the bio-assay of tetanus toxoids was discussed in the light of observations made.

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REFERENCES

1. GREENBERG, L., C. A. MORRELL, and J. GIBBARD. 1943. The biological assay of tetanus toxoid. *J. Immunol.* **46**: 333-340.
2. IPSEN, J. 1953. Bio-assay of four tetanus-toxoids (aluminium-precipitated) in mice, guinea pigs and humans. *J. Immunol.* **70**: 426-434.
3. MURATA, R., E. WADA, A. YAMAMOTO, and K. KUBOTA. 1960. Studies on the quantitative measurement of tetanus toxoids. Part II. The international unit of tetanus toxoid and its error determined by mice method (In Japanese). *Jap. J. Bact.* **15**: 866-870.
4. GREENBERG, L. 1953. International standard for tetanus toxoid. *Bull. Wld. Hlth. Org.* **9**: 837-842.
5. IPSEN, J. 1954. Inherent immunizability to tetanus toxoid, base on studies in pure inbred mice. *J. Immunol.* **72**: 243-247.
6. WADA, E., A. YAMAMOTO, S. HIROSE, M. TAKAO, Y. OHTA, S YAMAMOTO, Y. MIYASAKI, and M. KOBAYASHI. 1959. On the immunizability of various mice strain against tetanus toxoids (In Japanese). *Jap. J. Bact.* **14**; 546-551.
7. CHEN, B. L. and K. ZIA. 1962. Studies on secondary tetanus immunization in mice. I. Dose-response relationship. *Bull. Inst. Zool., Academia Sinica.* **1**: 47-53.
8. SCHEIBEL, I. 1957. Control of the Danish combined Diphtheria-tetanus vaccine with special reference to the assaying of its antigenic potency in international units. *Proceedings 3rd International Meeting of Biological Standardization.* p 153-163.
9. CHEN, B. L., T. J. YANG, M. L. PAN and K. ZIA. 1962. Scoring of tetanus immunity in mice. *Bull. Inst. Zool., Academia Sinica.* **1**: 55-61.
10. BURN, J. H., D. J. FINNEY and L. G. GOODWIN. 1952. *Biological Standardization.* Oxford University Press, London. p 48-59.
11. OSTLE, B. 1956. *Statistics in Research.* Iowa State College Press, Ames. p 129.
12. PRIGGE, R. 1935. Die staatliche Prufung der Diphtherie-Impfstoffe und ihre experimentellen Grundlagen. *Arb. Staatsinst. f. Exp. Therap.* **32**: 1-50.
13. IPSEN, J. 1953. Precision of potency assay

of alum-precipitated tetanus toxoid in mice. *J. Immunol.* **70**: 171-180.

14. IPSEN, J. 1952. The effect of environmental temperature on the immune response of mice to tetanus toxoid. *J. Immunol.* **69**: 273-283.
15. BARR, M. and M. LLEWELLYN-JONES. 1953. Some factors influencing the response of animals to immunization with combined prophylactics. *Brit. J. Exp. Path.* **34**: 12-22.
16. MURATA, R., K. KUBOTA, E. WADA, and A. YAMAMOTO. 1960. Studies on the quantitative measurement of tetanus toxoid. III. Difference in the immune response against tetanus toxoid between guinea pig and mouse (In Japanese). *Jap. J. Bact.* **15**: 1275-1279.
17. CHEN, B. L., C. T. CHOU, C. T. HUANG, Y. T. WANG, H. H. KO, W. C. HUANG, and C. F. LING. 1957. Studies on diphtheria-pertussis-tetanus combined immunization in children. III. Immune responses after the booster vaccination. *J. Immunol.* **79**: 393-400.